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A New mtDNA Mutation Associated with Leber Hereditary Optic Neuroretinopathy

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Summary

A single base mutation at nucleotide position 3460 (nt 3460) in the ND1 gene in human mtDNA was found to be associated with Leber hereditary optic neuroretinopathy (LHON). The G-to-A mutation converts an alanine to a threonine at the 52d codon of the gene. The mutation also abolishes an *Aha*II restriction site and thus can be detected easily by RFLP analysis. The mutation was found in three independent Finnish LHON families but in none of the 60 controls. None of the families with the nt 3460 mutation in ND1 had the previously reported nt 11778 mutation in the ND4 gene. The G-to-A change at nt 3460 is the second mutation so far detected in LHON.

Introduction

Leber hereditary optic neuroretinopathy (LHON) is a maternally inherited ocular disease resulting in bilateral visual loss in young adults. The concept of mitochondrial transmission of LHON was strongly supported when Wallace et al. (1988) found a nucleotide change at nucleotide position 11778 (nt 11778) (Anderson et al. 1981) in mtDNA associated with LHON. The G-to-A transition was in the gene encoding for the subunit 4 (ND4), one of the seven mitochondrially encoded subunits of the mitochondrial NADH–ubiquinone oxidoreductase (NADH dehydrogenase, or complex I). Subsequent studies by other groups (Holt et al. 1989; Vilkki et al. 1989*b*) showed that genetic heterogeneity in LHON is common, since about half of the families did not have the mutation.

We have since searched for alternative LHON mutations by sequencing the mitochondrial genes for NADH–ubiquinone oxidoreductase subunits (ND genes). When no mutations were found in the ND4 gene (Huoponen et al. 1990), we directed our interest to other ND genes and found a new mutation in the ND1 gene. A G-to-A base change at nt 3460 was found in three independent Finnish LHON families.

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Material and Methods

Our study material consisted of 10 Finnish LHON families that had earlier been shown not to have the ND4 mutation at nt 11778 (Wallace et al. 1988; Vilkki et al. 1989*b*). The clinical disease in the affected individuals was fully compatible with LHON. The clinical data of families 1 and 2 (termed families C and B, respectively, by Nikoskelainen et al. [1987]) have been published earlier. The presence of the new mutation was also studied in 11 Finnish LHON families that had the ND4 mutation.

The controls consisted of 60 maternally unrelated individuals with no known ocular disease. They represent 17 mtDNA types previously found in a Finnish population sample (Vilkki et al. 1989*a*).

DNA sequencing

Total DNA was extracted from blood samples according to a method described elsewhere (Vilkki et al. 1988). The DNA sequence was determined by direct sequencing of PCR-amplified mtDNA by using the dideoxy chain termination method of Sanger et al.

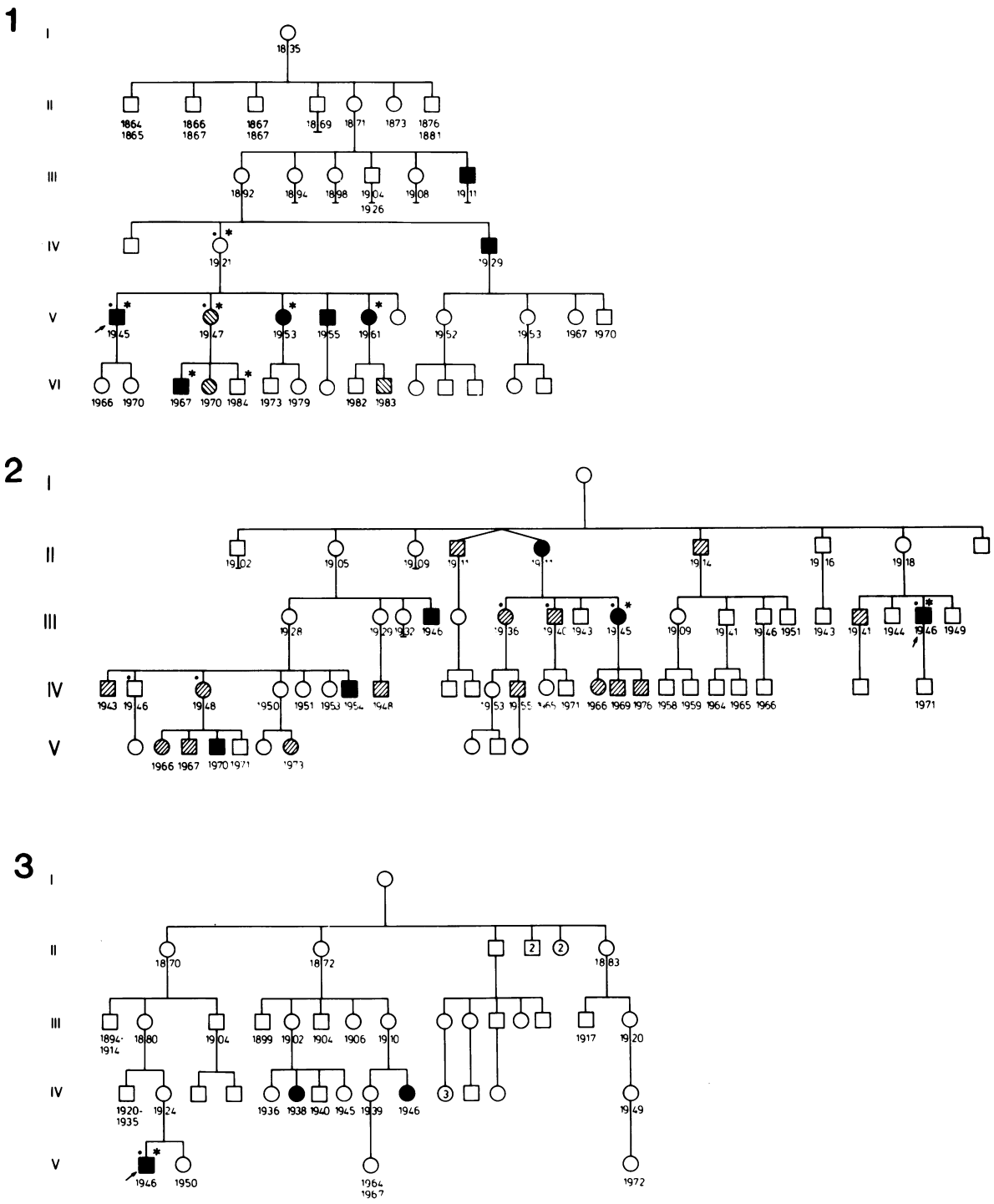

Figure 1 Pedigrees of three Finnish LHON families with mutation at nt 3460 in ND1 gene. The blackened symbols indicate individuals with optic atrophy; the cross-hatched symbols indicate individuals with peripapillary microangiopathy; and the open symbols indicate individuals with normal fundi. Individuals who have been subjected to DNA sequence analysis are marked with an asterisk, and individuals subjected to sequence-specific oligonucleotide hybridization are marked with a dot. The birth years are indicated below each individual. The probands are indicated by arrows.

(1977). The primers for PCR and sequencing were synthesized with PCR Mate DNA synthesizer (Applied Biosystems). For sequencing reactions a 1,290-bp fragment covering the entire ND1 gene was amplified with primers located at nt 3206–3226 (→) and nt 4496–4476 (←). The sequencing primers were located at nt 3426–3448, nt 3647–3666, nt 3874–3890, and nt 4092–4109. The primers used for PCR were also used as sequencing primers. The entire ND1 gene was sequenced from the affected probands of families 1 (V-1) and 2 (III-17) (fig. 1).

A 522-bp fragment encompassing the observed mutation site was amplified with primers located at nt 3206–3226 (→) and nt 3728–3708 (←). The region spanning the mutation site was sequenced in 10 individuals: three affected (V-3, V-5, and VI-3), two asymptomatic (IV-2 and VI-5), and one with microangiopathy (V-2) from family 1; one affected (III-9) from family 2; one affected (V-1) from family 3; and two normal controls. The PCR procedure and the sequencing reactions were carried out according to methods described elsewhere (Huoponen et al. 1990).

AhaII-RFLP analysis

For *Aha*II-RFLP analysis a 522-bp fragment encompassing the mutation site was amplified with primers located at nt 3206–3226 (→) and nt 3728–3708 (←). The amplification products were digested with *Aha*II (New England Biolabs) with 2 units enzyme/μg DNA under conditions recommended by the supplier. After digestion the samples were run in 1% agarose gel stained with ethidium bromide.

The *Aha*II-RFLP analysis was performed in one individual of each of the 11 families that had the ND4 mutation and in at least one individual of the families that did not have the ND4 mutation. In addition, 60 controls were analyzed.

Sequence-specific Oligonucleotide Hybridization

The probes used in the sequence-specific oligonucleotide hybridization were 5′-GAGTTTTATGG-CGTCAGCGAAGG-3′ for the normal sequence and 5′-CCTTCGCTGACACCATAAAACTC-3′ for the mutant sequence (opposite polarity was used to achieve the least stable mismatch pair). Duplicate samples of the PCR-amplified entire ND1 were electrophoresed through 1.5% agarose and were transferred onto Hybond N-membranes (Amersham).

The hybridizations were carried out for 12 h at 65°C in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS. The filters were washed twice in 2 × SSPE, 0.1% SDS at room temperature, followed by stringent wash at 70°C for the normal probe and at 68°C for the mutant probe. By this means we analyzed the PCR-amplified mtDNA samples of three non-LHON controls, two individuals with the nt 11778 mutation, one affected individual in each of the seven LHON families without known ND mutations, and 10 individuals from the three LHON families with the ND1 mutation (fig. 1).

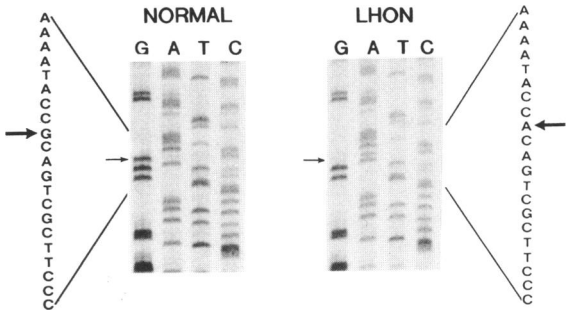

Figure 2 DNA sequence of region spanning mutation site at nt 3460 in ND1 gene of normal individual (*left*) and individual with LHON (*right*). The mutation site is indicated by an arrow.

Computer Analysis

The sequence was compared with the published sequences for bovine (Anderson et al. 1982), mouse (Bibb et al. 1981), rat (Gadaleta et al. 1989), and sea urchin (Cantatore et al. 1989) and with the GenBank Collection sequences for *Neurospora crassa, Drosophila yakuba, Xenopus laevis,* and *Locusta migratoria.* The analyses of the sequences were performed on a VAX computer using the University of Wisconsin software package.

Results

The mutation was initially detected by direct sequencing of the PCR-amplified ND1 gene in one member of each of two separate Finnish LHON families (V-1 in family 1 and III-17 in family 2) (fig. 1). The mutation was a G-to-A transition at nt 3460 (fig. 2).

This leads to an amino acid replacement, of an alanine by a threonine, at codon 52 in the ND1 gene of the mitochondrial NADH–ubiquinone oxidoreductase. The only other nucleotide alteration in the ND1 gene, compared with the published human sequence (Anderson et al. 1981), was a silent mutation, G to T, at nt 3423. The same nucleotide change has already been noted by Wallace et al. (1988).

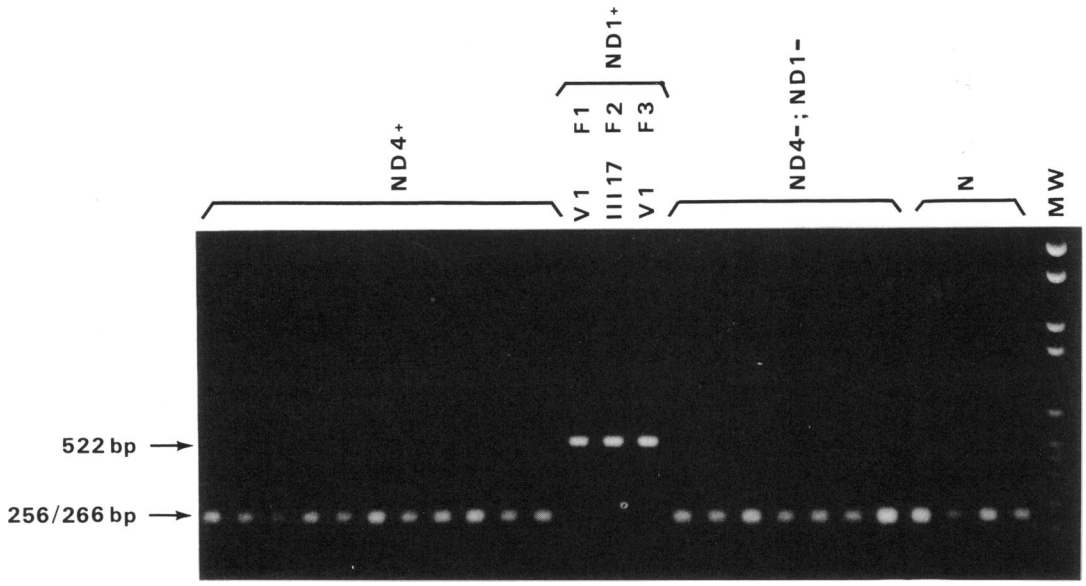

Figure 3 mtDNA mutation at position nt 3460, detected by *Aha*II analysis. In normal mtDNA, *Aha*II cuts the PCR-amplified 522-bp fragment to fragments of 256 bp and 266 bp, which comigrate in agarose gel. The mutation eliminates the *Aha*II restriction site and results in an intact 522-bp fragment. The lanes represent affected individuals from LHON families that have the ND4 mutation (ND4 +), affected members from the three LHON families that have the ND1 mutation (F1–F3;ND1 +), affected probands from LHON families that have no known mtDNA ND mutation (ND4 – ;ND1 –), maternally unrelated controls who have no ocular disease (N), and a molecular-weight marker (MW).

The base change at nt 3460 abolishes an *Aha*II restriction site, providing a rapid and simple test for detection of the mutation. *Aha*II treatment of PCR-amplified 522-bp fragments from normal mtDNA produced fragments of 256 bp and 266 bp, whereas from the mutated DNA the intact 522-bp fragment was obtained (fig. 3). The *Aha*II RFLP analysis indicated that, in addition to the two families (fig. 1, families 1 and 2) shown to have the mutation by sequencing, a third family with LHON also lacked the *Aha*II site (figs. 1 and 3). Altogether, the site was lacking in three Finnish LHON families, of which none have the ND4 mutation.

The site was present in 60 maternally unrelated controls and in 18 LHON families, 11 of which have the ND4 mutation. Thus, in seven families no mtDNA mutation associated with LHON has so far been detected.

The sequence change detected by *Aha*II screening was confirmed by sequencing the region spanning the mutation site. Six additional members of family 1, and one additional individual from family 2, and the proband from family 3 (fig. 1) showed the G-to-A change at nt 3460, whereas two controls showed G at nt 3460.

The presence of the mutation was further confirmed by sequence-specific oligonucleotide hybridization for several members of families 1 and 2 and for the proband of family 3. The sequence-specific oligonucleotide hybridizations, as shown in figure 4, revealed that the mutation was present in all 10 individuals belonging to families 1–3 and was not found either in the controls or in the members of other LHON families. No traces of normal mtDNA were observed in the mutant individuals, indicating that the nt 3460 mutation was homoplasmic. Conversely, the controls were homoplasmic for normal mtDNA (fig. 4).

Discussion

Our results indicate that the mtDNA mutation at nt 3460 in the ND1 is a second mutation associated with LHON. The mutation was present in three LHON families representing definite LHON and not having the previously known mtDNA mutation in ND4 (Wallace et al. 1988; Vilkki et al. 1989*b*). The mutation was not found in 60 maternally unrelated controls. Extensive genealogical studies of the families have not revealed any common ancestors. Furthermore, they represent two mtDNA types (termed types 1 and 11

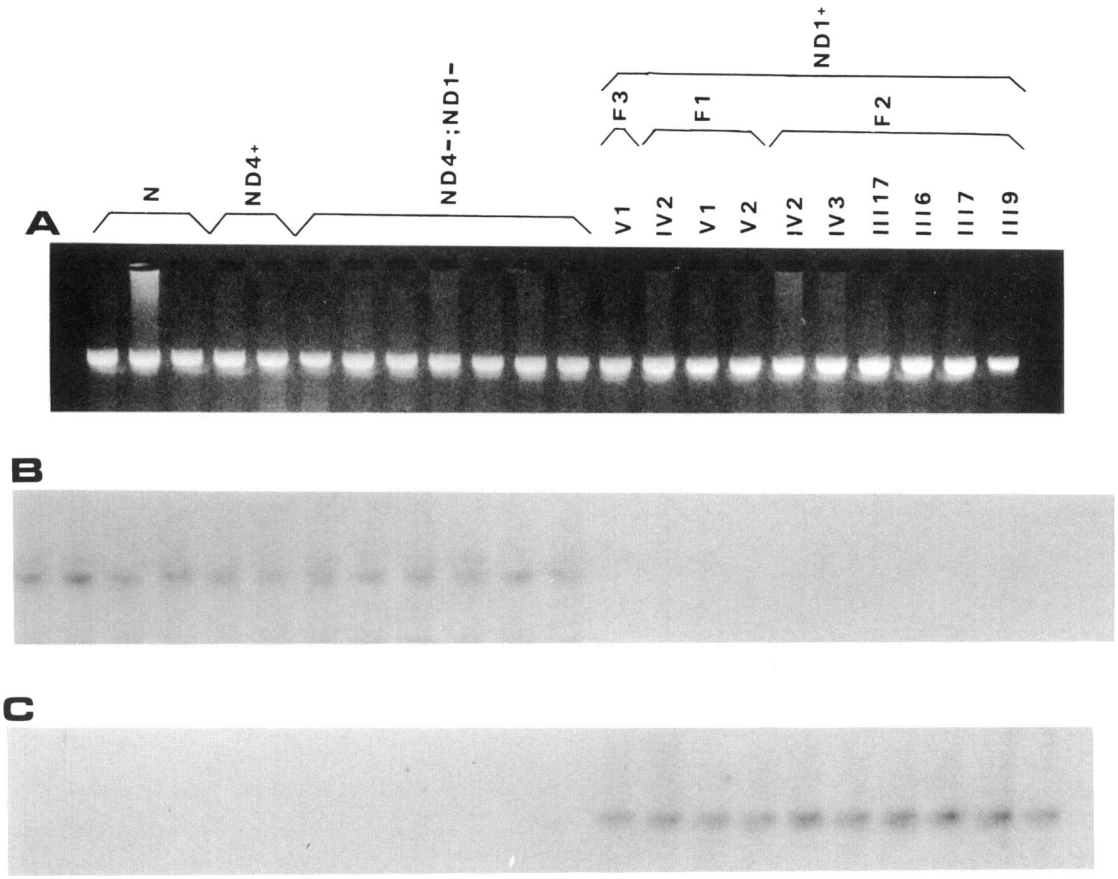

Figure 4 mtDNA analysis by sequence-specific oligonucleotide hybridization to PCR-amplified ND1 region. *A*, Ethidium bromide–stained 1.5% agarose gel of PCR-amplified 1,290-bp fragment encompassing ND1 gene. From left to right, the lanes represent three normal controls (N), two individuals who have the ND4 mutation (ND4 +), seven families that have no known ND mutation (ND4 − ;ND1 −), and 10 individuals from the three families that have the ND1 mutation (F1–F3,ND1 +). *B*, Southern blot of gel hybridized with labeled oligonucleotide complementary to normal sequence at nt 3460. No traces of normal mtDNA are seen in the individuals who have the ND1 mutation. *C*, Southern blot of gel hybridized with labeled oligonucleotide complementary to mutant sequence at nt 3460. Mutant mtDNA is detected only in the families that have the ND1 mutation.

by Vilkki et al. [1989*a*]), implying independent origin of the mutation. The ND1 gene is evolutionarily the most conserved of the ND genes, and there is evidence that its product may be intimately involved in the enzyme's function (Earley et al. 1987; Friedrich et al. 1990). The polypeptide is extremely rich in hydrophobic amino acids. The G-to-A transition at nt 3460 leads to a biochemically radical change of a hydrophobic alanine to a hydrophilic threonine in the NH_2 terminus of the polypeptide (Friedrich et al. 1990). Furthermore, the 52d amino acid residue is conserved, being hydrophobic in all species and alanine in most of them (table 1). The evolutionary conservation at codon 52 also provides evidence of the functional significance that the mutation has in the pathogenesis of LHON. The final proof for this, however, must await elucidation of biochemical changes due to the gene defect. At present, the biochemistry of LHON is largely unknown, not only in the present families that have the ND1 mutation but also in the families that have the ND4 mutation.

The mutation was present in individuals displaying varied phenotypes, ranging from normal fundi or microangiopathy to complete optic atrophy. Heteroplasmy, the presence of a mixture of mutant and normal mtDNA, has been suggested to explain some of the intrafamilial clinical variation in LHON (Lott et al. 1990). No evidence of heteroplasmy was found, however, in the present study's families that have the nt 3460 mutation. The possibility of heteroplasmy

Table 1

Alignment of Nucleotide Sequences of Region Spanning nt 3460 Mutation Site

Species	Nucleotide Sequence[a]					Amino Acid
Human (normal)	TTC	GCT	GAC	GCC	ATA	Ala
Human (LHON)	TTC	GCT	GAC	ACC	ATA	Thr
Bovine	CAC	GCC	GAT	GCA	ATC	Ala
Mouse	TTT	GCA	GAC	GCC	ATA	Ala
Rat	TTT	GCA	GAT	GCC	ATA	Ala
Sea urchin	TTT	GCA	GAC	GGC	CTT	Gly
Neurospora crassa	TTT	GCT	GAT	GCT	TTG	Ala
Drosophila yakuba	TTT	TGT	GAT	GCA	ATT	Ala
Xenopus laevis	ATT	GCA	GAT	GGA	GTA	Gly
Locusta migratoria	TTT	AGT	GAT	GCT	ATT	Ala

[a] The mutated base associated with LHON is underlined.

cannot be completely ruled out, because the target tissues—retina and optical nerve—are not available for analysis. Some of the clinical variation may be caused by interaction of mitochondrial and nuclear genes. Our recent linkage studies suggest that the expression of optic atrophy is controlled by a commonly occurring X-chromosomal allele located on the proximal Xp (Vilkki et al. 1991).

The ophthalmological findings in the affected individuals from the three families that have the ND1 mutation are similar to those in individuals from families that have the ND4 mutation. Detailed neurological and other clinical studies will be needed to find out whether the phenotypic manifestation of the different mutations can be distinguished.

Acknowledgments

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